FEBS Letters 586 (2012) 4186–4189

journal homepage: www.FEBSLetters.org

Crossover inhibition as an indicator of convergent evolution of enzyme mechanisms: A β-lactamase and a N-terminal nucleophile hydrolase

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ARTICLE INFO

Article history:
Received 29 August 2012
Revised 1 October 2012
Accepted 2 October 2012
Available online 23 October 2012

Edited by Stuart Ferguson

Keywords:
Enzyme
β-Lactamase
Proteasome
Inhibitors
Convergent evolution

ABSTRACT

O-Aryloxycarbonyl hydroxamates and 1,3,4-oxathiazol-2-ones have been identified as covalent inhibitors of β-lactamases and proteasomes, respectively. The products of these inhibition reactions are remarkably similar, involving carbonyl cross-linking of the active sites. We have cross-checked these inhibitors, showing that the former inhibit proteasomes and the latter β-lactamases, to form the same inactive carbonyl adducts. These results are discussed in terms of similarities of the active site structures and catalytic mechanisms. It is likely that a mechanistic imperative has led to convergent evolution of these enzyme active sites, of a β-lactam-recognizing enzyme and a N-terminal protease belonging to different amidohydrolase superfamilies.

1. Introduction

To a large degree, the resistance of bacteria to the β-lactam antibiotics derives from bacterial expression of β-lactamases [1]. These enzymes catalyze the hydrolysis of the β-lactam ring and thus inactivate the antibiotic. β-Lactamase inhibitors are of interest because they can be administered in combination with a β-lactam and thereby extend the clinical lifetime of the antibiotic [2].

We recently described the *O*-aryloxycarbonyl hydroxamates **1** as novel serine β-lactamase inhibitors [3,4]. They were found to first acylate the nucleophilic active site serine to generate **3**. Subsequently, the active site became irreversibly inactivated by cross-linking of the serine to an active site lysine as in **4** (Scheme 1).

Another potential antibiotic target is the protease of the bacterial proteasome [5]. This enzyme is inhibited by a series of 1,3,4-oxathiazol-2-ones **2** [6]. We noticed that the proposed mechanism of inhibition, first involving acylation of the nucleophilic threonine of the active site to form **3** and followed by formation of an inert complex **4**, appeared to have much in common with that of the β-lactamase with **1** (Scheme 1). Here also, in the inert complex **4**, the active site nucleophile is cross-linked to an adjacent amine, in this case to the N-terminal threonine amine.

Because of the similarity of the intermediates **3** and final products **4**, we decided to cross-check these inhibitors. In this paper, we report the reaction of the proteosome with **1**, serine β-lactamases with **2**, and discuss the results in terms of the similarity of the active site structure and mechanism between these two classes of enzymes.

2. Materials and methods

The *Enterobacter cloacae* P99 and TEM-2 β-lactamases were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. The *Mycobacterium tuberculosis* proteasome was over-expressed and purified as reported [7]. *O*-Aryloxycarbonyl hydroxamates and the 1,3,4-oxathiazol-2-ones were synthesized as described [4,6]. 5-Phenyl-1,3,4-dioxazolone (**9**) was prepared from the reaction of benzohydroxamic acid with phenyl chloroformate in the presence of imidazole [8]. All kinetics measurements were carried out in 20 mM MOPS buffer, pH 7.5 at 25 °C (β-lactamases) and 20 mM HEPES, 0.5 mM EDTA, pH 7.5 at 37 °C (proteasome). Second order rate constants for inactivation (k_i) were determined as described [4,6] from measurements of enzyme activity (initial velocity) against 0.2 mM cephalothin (β-lactamase) or 50 μM Suc-LLVY-AMC (proteasome) as a function of time. Figs. 1A and B were generated from crystal structure files from the RCSB Protein Data Bank (1MXO, β-lactamase and 2FHH, proteasome) with the aid of Insight II (Accelrys) software. The β-lactamase electrospray mass spectra were obtained from the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois and the proteasome

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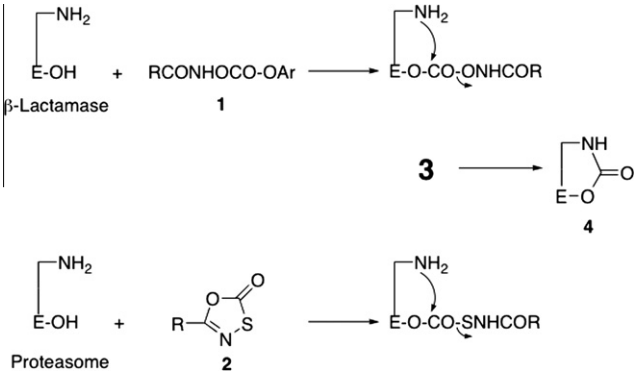
0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.10.019



Scheme 1. β-Lactamase and proteasome inhibition mechanisms.

peptide spectra with the assistance of Dr. Haiteng Deng at the Proteomics Resource Center of the Rockefeller University.

3. Results

The class C *Enterobacter cloacae* P99 and Class A TEM-2 β-lactamases were indeed inactivated by the 1,3,4-oxathiazol-2-ones **5**–**8** and the 1,3,4-dioxazol-2-one **9.** Second-order rates constants for loss of activity were determined as described in methods and the results are presented in Table 1. For comparison, rate constants for the *O*-aryloxycarbonyl hydroxamates **10–12** are included. Further, all of **5**–**12** also inhibit the proteasome (Table 1).

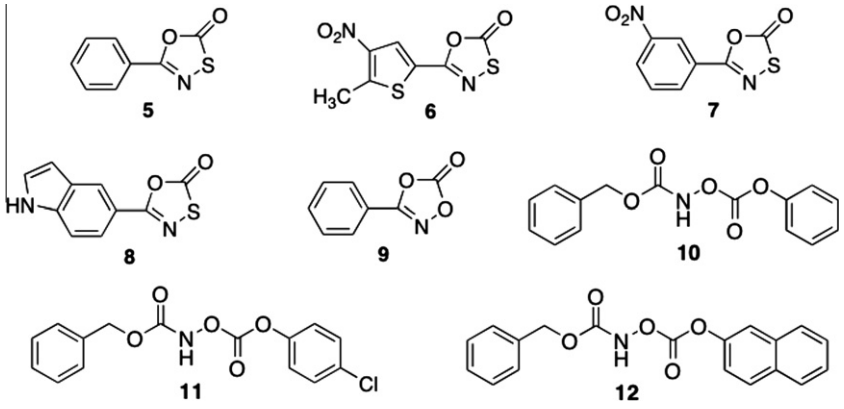
These data show that the 1,3,4-oxathiazol-2-ones are comparably effective to *O*-aryloxylcarbonyl hydroxamates as β-lactamase and proteasome inhibitors. Neither class of inhibitor, of course, is optimized for the particular enzymes of Table 1. It is striking, however, that **6** and **7** are so much better inhibitors of the P99 β-lactamase, and most likely of the TEM-2 enzyme also, than **5** and **8**. This cannot be due to intrinsic reactivity since the same difference is not seen in the background hydrolysis rates k_o, also presented in Table 1. It may reflect the benefit of an electrophilic aromatic ring in binding to the β-lactamase active site. Compounds **6** and **7** are also effective proteasome inhibitors, possibly for similar reasons. Incorporation of either a better (**11**) or more hydrophobic (**12**) leaving group into **10** leads to a more effective inhibitor of both the β-lactamases and the proteasome. It is striking that the 1,3,4-dioxazolone **9**, a direct oxa analog of **5**, is a weak/non-inhibitor of both types of enzyme, despite its higher reactivity (see k_0, Table 1). This probably results from partitioning of a tetrahedral intermediate in favor of free enzyme rather than **3** and thus **4** (Path a of Fig. 3b in Ref. [6]).

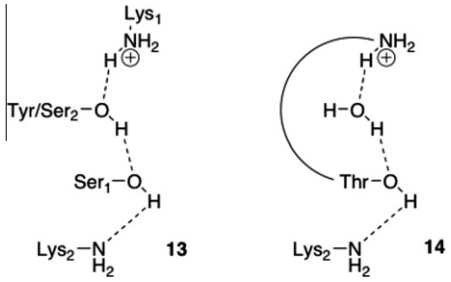
Mass spectra and crystal structures suggested that these inhibitors covalently modify both enzymes as described above (Scheme 1) [3,4]. A mass spectrum of the P99 β-lactamase after inactivation by **6** ($\Delta m/s$ = 26 amu) and of the N-terminal heptapeptide of the proteasome by **10** ($\Delta m/s$ = 26.0 amu) showed carbonyl addition, presumably by the same cross-linking mechanism.

4. Discussion

It is interesting, given this crossover of inhibitors with analogous mechanisms, described above, to consider a possible overlap of functional groups at the active sites of a β-lactamase and the proteasome. Could this reveal a common active site architecture and a common mechanism of catalysis?

Crystal structures of class C β-lactamases [2,9] and the yeast or *M. tuberculosis* proteasomes [7,10] show the active site components of **13** and **14**, respectively [7,11,12]. Hydrogens have been added to give structures poised for the first step of catalysis, nucleophilic attack on a substrate carbonyl, by the active site serine (β-lactamase) or threonine (proteasome), as prescribed by several of the commonly-held mechanisms [4,5]; in each case, nucleophilic attack by the alcohol is aided by a lysine ε-amine as general base. It is interesting to note that the tyrosine hydroxyl of the β-lactamase is replaced in the proteasome structure by that of a water molecule. Structures similar to **13** are seen in the DD-peptidases, the evolutionary parents of β-lactamases [13,14] and in all proteasomes. On the other hand, other major groups of N-terminal hydrolases, such as the penicillin acylases [15], do not appear to contain Lys_2 [16]. These two groups of enzymes, the β-lactam-recognizing enzymes and the N-terminal hydrolases, belong to two very different superfamilies, from the point of view of tertiary structure [16–18]. It may be noted, however, that another superfamily of serine amidohydrolases, that containing the amidase signature enzymes, has a very similar array of active site functional groups [19].





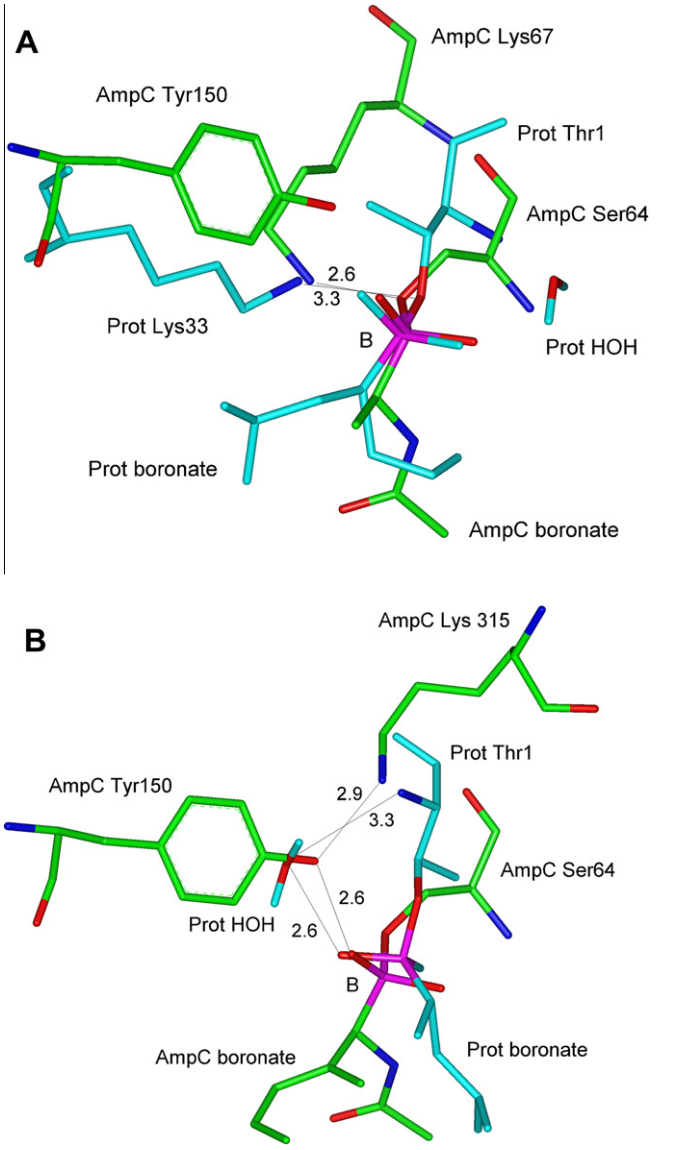


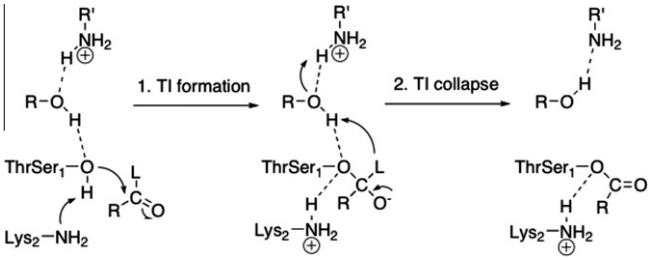
Fig. 1. (A) Superimposition of active site elements of a boronate transition state analog structure of the class C AmpC β-lactamase (atomic coloring) with the C subunit of the *M. tuberculosis* proteasome (turquoise, with important heteroatoms atomic colored). The central boron (B) is shown in purple in each case. Only heavy atoms are shown except for hydrogens on the proteasome water molecule. Superimposed in this diagram (see text) are Ser/ThrOγBLys$_2$N (RMSD 0.33 Å), the tetrahedral intermediate formation elements (see Scheme 3), from each structure. (B) As above, with OBO(TyrO)Lys$_1$N of the β-lactamase superimposed on OBO(H$_2$O)ThrN of the proteasome (see text) (RMSD 0.68 Å), the tetrahedral intermediate breakdown elements (see Scheme 3).

A common acylation mechanism (Scheme 2) can then be proposed for the β-lactam recognizing enzymes (class C β-lactamases and DD-peptidases) and the proteasome (Scheme 2). [Note that class A and D β-lactamases, also serine enzymes, have additional features in the active site to assist turnover of β-lactams; they are therefore not included in this analysis]. These mechanisms are supported by crystal structures of complexes of the respective enzymes with boronic acid transition state analogs [7,14]. It should be noted, however, that a mechanism of proteasome catalysis employing the N-terminal threonine amine directly as a general base, i.e. omitting the intervening water molecule, has also been suggested and there is a boronic acid complex structure supporting this idea [20]. It is possible that both mechanisms may be available to the enzyme, depending on the structure of the peptide substrate.

Table 1
Rate constants of β-lactamase and proteasome inhibition.

Inhibitor	k_i (M^{-1} s^{-1})			$k_o \times 10^5$ s^{-1}
	P99 β-Lactamase	TEM-2 β-Lactamase	Mtb proteasome	
5	31 ± 2	<0.7	409[b]	1.2
6	2800 ± 300	0.7 ± 0.1	2130[b]	2.0
7	2200 ± 200	ND	730[b]	1.1
8	13.0 ± 0.6	ND	4.3[b]	1.9
9	0.23 ± 0.05	ND	NI	239
10	6.1×10^3[a]	13.4 ± 0.2	300 ± 20	25[a]
11	2.2×10^4[a]	66.2 ± 0.2	1160 ± 120	98[a]
12	1.8×10^4[a]	25.0 ± 0.2	2040 ± 160	45[a]

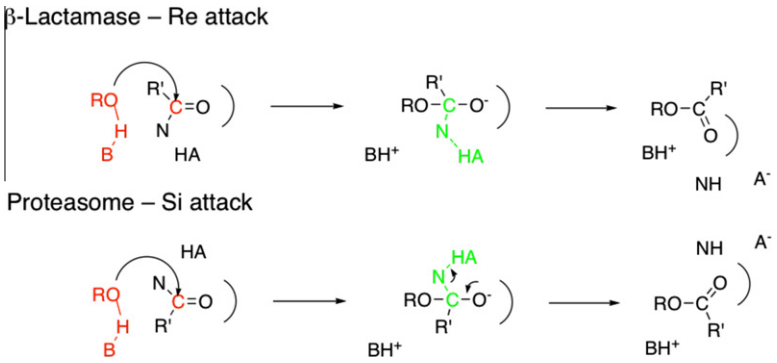
ND = not determined, NI = no inhibition.
[a] Ref. [4].
[b] Ref. [6].



Scheme 2. A possible common enzyme acylation mechanism.

The tetrahedral intermediates formed from reaction of normal substrates with each enzyme in Scheme 2 are stabilized by an oxyanion hole, composed of backbone NH hydrogen bond donors [7,14]. The tetrahedral intermediates in the reactions of **10** with the class C β-lactamase, however, were proposed to be stabilized by Tyr 150, the tyrosine depicted in **13**, as an "alternative oxyanion hole" [4]. The proteasome analog of the tyrosine hydroxyl would be the water molecule of **14**.

A significant difference, however, evident from the boronate crystal structures [7,14], between class C β-lactamases and DD-peptidases on one hand and the proteasome on the other, reflecting their very different evolutionary heritage, is that the former attack the substrate (peptide) carbonyl on the Re face of the substrate carbonyl, generating a S tetrahedral intermediate, while the proteasome threonine attacks on the Si face to generate an R intermediate (Scheme 3). For this reason, the active site components of **13** and **14** (otherwise seen as the red and green elements of Scheme 3) are differently arranged in space and cannot be directly superimposed. Separately, however, the functional groups relevant to the two steps of Scheme 2 can be superimposed. Thus, the Lys$_2$s of **13** and **14**, each putatively participating as a general base in the first step of the acylation reaction (Scheme 2; B of Scheme 3), can be superimposed [OγBLys$_2$N atoms of the two structures, RMSD 0.33 Å] as shown in Fig. 1A. It is then noticeable that Tyr 150 of the β-lactamase and the H$_2$O of the proteasome are on opposite sides of the boronate, hydrogen-bonded to stereochemically distinct boronate oxygens. The simplicity of **13** and **14** remains, however, since the general acid elements of Scheme 2 (HA of Scheme 3) can also be superimposed. Thus, OBO(TyrO)Lys$_1$N of the β-lactamase can be superimposed on OBO(H$_2$O)ThrN of the proteasome (RMSD 0.68 Å) (Fig. 1B). These are the functional groups putatively involved in the second step of the acylation reaction, facilitating departure of the leaving group (Scheme 2). It seems likely therefore, that a mechanistic imperative has forced convergent evolution of the active site functional groups of the



Scheme 3. Participants in the two steps, acylation, red, and deacylation, green, cannot be superimposed all together but each can be seperately, as in Fig. 1A and B.

β-lactam-recognizing enzymes and the proteasome, despite their different stereochemistry.

The stereochemistry of nucleophilic attack at an amide substrate carbonyl group varies among amidohydrolases. Two enzymes with the same array of active site functional groups but with different stereochemistries of attack (and usually with different protein folds) are usually thought to arise from convergent evolution. For example, the serine carboxypeptidases of Clan SC, Family S10 [21] attack the Si face while the classical serine proteases of Clan PA, Family S1 attack the Re [22]. The catalytic triad is, however, believed to act in the same way in each family. A number of serine/lysine amidohydrolases, including the proteasome, as noted above, attack the Si face [23] while others, including the β-lactam-recognizing enzymes and amidase signature enzymes approach the Re face [19].

With respect to Scheme 2, it should be noted, as has been previously discussed for β-lactamases [14], that alternative mechanisms of hydrolysis can be proposed on the basis of crystal structures of boronate complexes. For example, the roles of the two lysines in Scheme 2 can be reversed, so that Lys_1 (RNH_2) is the indirect general base for nucleophilic attack by Ser_1 (Thr) and a protonated Lys_2 facilitates leaving group departure. The superimpositions suggested by this mechanism, however, are not as close as those in Fig. 1.

A cross-check of inhibitors, the *O*-aryloxycarbonyl hydroxamates, **1**, and the 1,3,4-oxathiazol-2-ones, **2**, shows that they have a very similar mechanism of inhibition of β-lactamases and the proteasome. These enzymes are representative members of two distinct protein superfamilies, the β-lactam-recognizing enzymes and N-terminal hydrolases, respectively. Examination of relevant protein crystal structures has demonstrated the presence of a very similarly placed array of analogous active site functional groups, two amines and two hydroxyl groups, in these two groups of enzymes. A very similar catalytic mechanism for amide hydrolysis is thus indicated. We now see that these common elements of active site structure and mechanism are available to three serine amidohydrolase superfamilies, the two above and the amidase signature enzymes [19]. The relationship between the active sites of the above enzymes and those of other serine amidohydrolase superfamilies, for example the classical serine proteases, has been previously discussed [19].

Acknowledgments

We acknowledge the support and encouragement of Dr Carl F. Nathan of the Department of Microbiology and Immunology, Weill Cornell Medical College, New York, New York. This research was supported by the National Institutes of Health (RFP, AI017986 and CN, AI080618). We also acknowledge the assistance of Dr. Huilin Li and Dr. Shaoqing Yang at the Brookhaven National Laboratory with structural studies.

References

[1] Frére, J.-M., Ed., (2012). Beta-Lactamases, Nova, NY.
[2] Drawz, S.R. and Bonomo, R.A. (2009) Three decades of beta-lactamase inhibitors. Clin. Microbiol. Rev. 1, 160–201.
[3] Wyrembak, P.N., Babaoglu, K., Pelto, R.B., Shoichet, B.K. and Pratt, R.F. (2007) *O*-Aryloxycarbonyl hydroxamates: new β-lactamase inhibitors that cross-link the active site. J. Am. Chem. Soc. 129, 9548–9549.
[4] Pelto, R.B. and Pratt, R.F. (2008) Kinetics and mechanism of inhibition of a serine β-lactamase by *O*-aryloxycarbonyl hydroxamates. Biochemistry 47, 12037–12046.
[5] Borissenko, L. and Groll, M. (2007) 20S proteasome and its inhibitors: crystallographic knowledge for drug development. Chem. Rev. 107, 687–717.
[6] Lin, G., Li, D., de Carvalho, L.P.S., Deng, H., Tao, H., Vogt, G., Wu, K., Schneider, J., Chidawanyika, T., Warren, J.D., Li, H. and Nathan, C. (2009) Inhibitors selective for mycobacterial versus human proteasomes. Nature 461, 621–626.
[7] Hu, G., Lin, G., Wang, M., Dick, L., Xu, R.-M., Nathan, C. and Li, H. (2006) Structure of the *Mycobacterium tuberculosis* proteasome and mechanism of inhibition by a peptidyl boronate. Mol. Microbiol. 59, 1417–1428.
[8] Wyrembak, P.N. (2003) *N*,*O*-Diacyl hydroxylamines as β-lactamase inhibitors. BA Thesis, Wesleyan University.
[9] Kelly, J.A. and Kuzin, A.P. (1995) The refined crystallographic structure of a DD-peptidase penicillin-target enzyme at 1.6 Å resolution. J. Mol. Biol. 254, 233–236.
[10] Groll, M., Ditzel, L., Löwe, J., Stock, D., Bochtler, M., Bartunik, H.D. and Huber, R. (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. Nature 386, 463–471.
[11] Matagne, A., Dubus, A., Galleni, M. and Frére, J.-M. (1999) The β-lactamase cycle: a tale of selective pressure and bacterial ingenuity. Nat. Prod. Rep. 16, 1–19.
[12] Fernollar-Ferrer, C., Frau, J., Donoso, J. and Munoz, F. (2008) Evolution of class C β-lactamases: factors influencing their hydrolysis and recognition mechanism. Theor. Chem. Acc. 121, 209–218.
[13] Sauvage, E., Kerff, F., Terrak, M., Ayala, J.A. and Charlier, P. (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol. Rev. 32, 234–258.
[14] Dzhekieva, L., Rocaboy, M., Kerff, F., Charlier, P., Sauvage, E. and Pratt, R.F. (2010) Crystal structure of a complex between the *Actinomadura* R39 DD-peptidase and a peptidoglycan-mimetic boronate inhibitor: interpretation of a transition state analogue in terms of catalytic mechanism. Biochemistry 49, 6411–6419.
[15] Duggleby, H.J., Tolley, S.P., Hill, C.P., Dodson, E.J., Dodson, G. and Moody, P.C.E. (1995) Penicillin acylase has a single amino acid catalytic centre. Nature 373, 264–268.
[16] Oinonen, C. and Rouvinen, J. (2000) Structural comparisons of Ntn-hydrolases. Protein Sci. 2, 2329–2337.
[17] Galperin, G.Y. and Koonin, E.V. (2012) Divergence and convergence in enzyme evolution. J. Biol. Chem. 287, 21–28.
[18] Goffin, C. and Ghuysen, J.-M. (1998) Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol. Mol. Biol. Rev. 62, 1079–1083.
[19] Pratt, R.F. and McLeish, M.J. (2010) Structural relationship between the active sites of β-lactam-recognizing and amidase signature enzymes: convergent evolution? Biochemistry 49, 9688–9697.
[20] Groll, M., Berkers, C.R., Ploegh, H.L. and Ovaa, H. (2006) Crystal structure of the boronic acid-based proteasome inhibitor bortezomib in complex with the yeast 20S proteasome. Structure 14, 451–458.
[21] Rawlings, N.D., Barrett, A.J. and Bateman, A. (2010) MEROPS: the peptidase database. Nucleic Acids Res. 38, D227–D233.
[22] Bullock, T.A., Breddam, K. and Remington, S.J. (1996) Peptide aldehyde complexes with wheat serine carboxypeptidase II: implications for the catalytic mechanism and substrate specificity. J. Mol. Biol. 255, 714–725.
[23] Paetzel, M., Dalbey, R.E. and Strynadka, N.C.G. (1998) Crystal structure of a bacterial signal complex with a β-lactam inhibitor. Nature 396, 186–190.